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(54) **Process for producing trehalose derivatives**

(57) High quality trehalose derivatives are readily prepared in a considerably high yield by reacting anhydrous trehalose with reactive reagents under anhydrous conditions. The trehalose derivatives can be used in a variety of fields in the production, the chemical synthe-

sis, and the enzymatic synthesis of foods, cosmetics, pharmaceuticals, detergents and chemicals as surfactants, humectants, skin-beautifying agents, antitumor agents, and intermediates for chemical and enzymatic syntheses.

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## Description

The present invention relates to a novel preparation of trehalose derivatives, more particularly, to a preparation of trehalose derivatives characterized in that it comprises a step of allowing a reactive reagent to react with anhydrous trehalose under anhydrous conditions.

Recently, acid esters and alkyl ethers of trehalose such as fatty acid esters and sulfonic acid esters of trehalose are highlighted. Nishikawa et al. reported in *"The Chemical Society of Japan"*, No.10, pp.1,661-1,666 (1982) that trehalose esters of stearic acid, palmitic acid, and myristic acid strongly inhibited the growth of malignant tumors both *in vivo* and *in vitro*, and implied that these derivatives might be effective as antitumor agents. For example, Japanese Patent Laid-Open No.290,808/92 discloses that trehalose sulfates satisfactorily retain the water valance of the skin and impart an appropriate moisture to the skin. Therefore, these sulfates can be incorporated in some cosmetics as satisfactory humectants and skin-whitening agents. Trehalose ethers with alkyls of 8-25 carbon atoms are known as useful surfactants with a relatively high safeness and activity.

As is disclosed by C. K. Lee in *"Developments in Food Carbohydrate-2"*, pp.1-89 (1980), published by Applied Science Publishers, and K. Yoshimoto in *"Chemical and Pharmaceutical Bulletin"*, Vol.30, No.4, pp.1,169-1,174 (1982), trehalose derivatives are generally prepared by reacting reactive reagents with crystalline trehalose hydrate under anhydrous conditions, and the quality and the yield of the final products especially depend on whether the moisture in the reaction systems can be eliminated to the highest possible level. However, even when prepared into a solid form, crystalline trehalose hydrate generally contains about 10 w/w % moisture and only results in an unsatisfactory quality and poor yield when used intact. Therefore, crystalline trehalose hydrate is dried up to an anhydrous state by using a dehydrant such as diphosphorus pentaoxide, prior to use, but the removal of the water of crystallization is substantially difficult. Thus, satisfactorily high quality of trehalose derivatives could not be readily obtained in a considerably high yield and low cost.

In view of the foregoing, the present invention aims to provide a preparation of trehalose derivatives with a relatively high quality and in a considerably high yield and low cost.

Accordingly, the present invention provides a process which comprises a step of allowing a reactive reagent to react with anhydrous trehalose under anhydrous conditions.

The present invention will now be described in further detail, by way of example only, with reference to the following Experiments and Examples.

The anhydrous trehalose used as a substrate in the present invention is substantially free from moisture. Therefore, trehalose derivatives with a relatively high quality can be readily obtained in a considerably high yield by using such anhydrous trehalose intact or after slight pre-drying.

Explaining now the present invention with reference to the following Experiments and Examples, the wording "trehalose derivatives" as referred to in the present specification includes those in general such as esters, ethers, halides, nitrogen-containing derivatives, and sulfur-containing derivatives of trehalose which are generally obtained by allowing reactive reagents to react with anhydrous trehalose under anhydrous conditions. The wording "reactive reagents" as referred to in the present specification means acids, salts, alcohols, ketones, halogens, amines, and reactive derivatives thereof which can form the present trehalose derivatives when reacted with anhydrous trehalose under anhydrous conditions. The present invention is applicable to chemical reaction systems in general where the moisture in the systems lowers the reactivity of reactive reagents, causes unsatisfactory side reactions, inhibits the main reactions, and results in the deterioration of the quality and/or the reduction of the yield of the objective trehalose derivatives, as well as in the increment of their production cost.

The wording "anhydrous trehalose" as referred to in the present specification means anhydrous crystalline and anhydrous amorphous trehaloses which are substantially free from moisture, i.e. those which contain a moisture less than 3 w/w % when determined by the Karl Fischer's method. Such anhydrous trehalose can be also used in the present invention, and anhydrous crystalline trehalose is particularly useful because it generally contains a relatively high trehalose content and is obtainable in a relatively low cost. Depending on the reaction types and the uses of trehalose derivatives, the higher the trehalose content in anhydrous trehalose the more satisfactory for the present invention: Generally, anhydrous trehalose with a trehalose content of 70 w/w % or higher, preferably, 80 w/w % or higher, on a dry solid basis (d.s.b.) can be satisfactorily used.

Among anhydrous trehalose, anhydrous amorphous trehalose can be obtained by dissolving trehalose in a small amount of water, and lyophilizing the aqueous solution, or by drying it with spray drying, etc, at a temperature exceeding the melting point of anhydrous crystalline trehalose, usually, at a temperature over 100°C. For example, Japanese Patent Laid-Open No.170,221/94 discloses a preparation of anhydrous crystalline trehalose which comprises concentrating a saccharide composition containing at least 60 w/w % trehalose, d.s.b., to give a moisture content less than 10 w/w %, preferably, into a syrupy product with a moisture content of higher than 2.0 w/w % but less than 9.5 w/w %, adding to the syrupy product 0.01-20 w/w %, d.s.b., of anhydrous crystalline trehalose as a seed crystal, keeping the contents at a temperature of 40-140°C to crystallize trehalose, and collecting the formed anhydrous crystalline trehalose

from the resultant masseccuite, or drying the masseccuite into a solid product. Generally, the anhydrous trehalose thus obtained does not substantially contain moisture, i.e. it only contains a moisture less than 3 w/w %.

The source and the origin of trehalose used as a material for anhydrous trehalose usable in the present invention are not specifically restricted to. Any one of those from cells of yeasts, those obtained by allowing complex enzyme systems comprising maltose- and trehalose-phosphorylases to act on maltose, those obtained by allowing maltose/trehalose converting enzymes to act on maltose to directly convert it into trehalose, and those obtained by enzymatically saccharifying partial starch hydrolysates. With a view point of producing satisfactorily-high quality trehalose derivatives in a relatively low cost, material trehaloses obtained by the aforesaid third and fourth methods are satisfactorily used.

To produce trehalose from starch, non-reducing saccharide-forming enzymes, as disclosed in Japanese Patent Application Nos. 349,216/93, 90,705/94, 166,011/94 and 190,183/94, are allowed to act on reducing partial starch hydrolysates, which contain maltooligosaccharides with a glucose polymerization degree of at least 3 such as maltotriose, maltotetraose, maltopentaose and maltohexaose obtained by gelatinizing and liquefying starch with acids and/or  $\alpha$ -amylase, to convert the maltooligosaccharides into non-reducing saccharides having a trehalose structure as an end unit. The non-reducing saccharides are subjected to the action of trehalose-releasing enzymes as disclosed in Japanese Patent Application Nos. 59,834/94, 79,291/94, 166,126/94 and 190,180/94 to release trehalose from the saccharides. In this case, these different types of non-reducing saccharide-forming enzymes and trehalose-releasing enzymes can be used simultaneously or sequentially, and the combination use of these two different types of enzymes and debranching enzymes such as isoamylase and pullulanase can more increase the trehalose content in the reaction mixtures. To directly convert maltose into trehalose, maltose/trehalose converting enzymes as disclosed, for example, in Japanese Patent Application Nos. 144,092/94, 156,399/94, 187,901/94 and 260,984/94, are allowed to act on maltose or saccharide compositions containing maltose. These specifications disclose processes for producing trehalose using maltose/trehalose converting enzymes, and any one of which can be used to produce the anhydrous trehalose used in the present invention. When a more highly purified trehalose is needed, the reaction mixtures obtained by those methods can be subjected to column chromatography using strong-acid cation exchangers in fixed-bed, moving-bed, or pseudo moving-bed methods to collect trehalose-rich fractions. The compositions and the fractions thus obtained contain at least 70 w/w % trehalose, d.s.b., and can be suitably used as a material for anhydrous trehalose.

Conventional methods can be used to react reactive reagents with anhydrous trehalose under anhydrous conditions. For example, methods as disclosed by C. K. Lee in *"Developments in Food Carbohydrate"*, pp. 1-89 (1980), published by Applied Science Publishers, and by K. Yoshimoto et al. in *"Chemical and Pharmaceutical Bulletin"*, Vol. 30, No. 4, pp. 1,169-1,174 (1982), as well as those disclosed in *"Carbohydrates as Organic Raw Materials"* (1991), published by VCH Publishers, New, York, USA, can be used in the present invention, and appropriately chosen to meet to the objective trehalose derivatives.

Explaining now briefly the representative methods for producing trehalose derivatives, carbonic acid esters of trehalose such as trehalose acetate and trehalose benzoate can be obtained by reacting anhydrous trehalose with its corresponding acid anhydrides or acid halides in alkaline organic solvents such as pyridine. To produce trehalose sulfate, anhydrous trehalose is allowed to react with complexes of sulfur trioxide and dimethyl sulfoxide or pyridine in a stream of inert- or rare-gases. Fatty acid esters of trehalose, prepared by reacting trehalose with fatty acids such as lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid, can be obtained by the condensation reaction of anhydrous trehalose and these fatty acids in the presence of alkaline catalysts or by reacting anhydrous trehalose with its corresponding fatty acid halides. Ethers of trehalose can be obtained by reacting anhydrous trehalose with excessive amounts of its corresponding alcohols in the presence of acid catalysts or by reacting anhydrous trehalose with its corresponding alkyl halides in the presence of alkaline catalysts.

Depending on their use, the trehalose derivatives thus obtained are usually used intact after removing the remaining reactive reagents and/or solvents by filtration, extraction, separation, separatory sedimentation, dialysis and distillation. If high-purity trehalose derivatives are needed, the intact or the partially purified trehalose derivatives can be treated with conventional methods used in this field such as thin layer chromatography, column chromatography, ion-exchange column chromatography, gas chromatography, distillation, and crystallization which are used to purify saccharides and saccharide derivatives. Two or more of these purification methods are used in combination to meet to final products. As is well known, trehalose gives 8 reactive functional groups in the normal substitution reaction where non-anomeric hydroxyl groups act mainly. This means that reaction products may contain a variety of trehalose derivatives with different substitution degrees depending on the reaction types and conditions. Usually, these products can be used intact and, if necessary, may be treated with one or more of the aforesaid purification methods to isolate the desired component(s).

The trehalose derivatives obtained by the present process can be widely applicable to the fields of food-, cosmetic- and pharmaceutical-industries. The fatty acid esters and alkyl ethers of trehalose are relatively high in activity, and useful as a surfactant for foods, cosmetics and pharmaceuticals. Depending on the types of fatty acids, the corresponding trehalose derivatives are expected to use as antioncotic agents. Trehalose sulfates can be incorporated into cosmetics as humectants or skin-whitening agents, while trehalose halides are useful as intermediates for synthesizing a

variety of derivatives.

The following experiments explain the effect and function of the present invention:

#### Experiment 1

##### Preparation of enzyme

##### Experiment 1-1

##### Preparation of non-reducing saccharide-forming enzyme

One hundred ml aliquots of a liquid nutrient culture medium (pH 7.0) containing 2.0 w/v % maltose, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate, and 0.1 w/v % sodium dihydrogen phosphate were placed in 500-ml Erlenmeyer flasks, and sterilized by autoclaving at 120°C for 20 min. After cooling the flasks, a seed of *Rhizobium* sp. M-11 (FERM BP-4130) was inoculated into each medium and incubated at 27°C for 24 hours under rotatory shaking conditions to obtain a seed culture. Thereafter, 20 L aliquots of a fresh preparation of the same nutrient culture medium were placed in 30-L jar fermenters, sterilized, and inoculated with one v/v of the seed culture, followed by the incubation at a pH of 6-8 and at 30°C for 24 hours under aeration-agitation conditions.

An about 18 L of the resultant culture was placed in a super high-pressure cell disrupting apparatus to disrupt cells, and the suspension was centrifuged to obtain a supernatant. To about 16 L of the supernatant was added ammonium sulfate to give a saturation degree of 20 w/v %, and the solution was allowed to stand at 4°C for an hour and centrifuged to remove sediment. The supernatant thus obtained was mixed with ammonium sulfate to give a saturation degree of 60 w/v %, allowed to stand at 4°C for 24 hours, centrifuged to collect sediment which was then dissolved in a minimum amount of 10 mM phosphate buffer (pH 7.0), dialyzed against 10 mM phosphate buffer (pH 7.0) for 24 hours, and centrifuged to remove insoluble substances. The resultant supernatant was fed to a column packed with "DEAF-TOYOPEARL®", an ion exchanger commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM phosphate buffer (pH 7.0), and eluted from the column with a linear gradient of sodium chloride creasing from 0 M to 0.5 M in 10 mM phosphate buffer (pH 7.0). Fractions with an enzymatic activity were collected from the eluate, pooled, dialyzed against 50 mM phosphate buffer (pH 7.0) containing 2 M ammonium sulfate for 10 hours, and centrifuged to remove insoluble substances. The resultant supernatant was fed to a column packed with "BUTYL-TOYOPEARL®", a gel for hydrophobic column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 50 mM phosphate buffer (pH 7.0) containing 2 M ammonium sulfate, and eluted from the column with a linear gradient of sodium chloride decreasing from 2 M to 0 M in 50 mM phosphate buffer (pH 7.0). Fractions with an enzymatic activity were collected from the eluate, pooled, fed to a column packed with "TOYOPEARL® HW-55", a gel for gel filtration column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 50 mM phosphate buffer (pH 7.0), and eluted from the column with 50 mM phosphate buffer (pH 7.0) to collect fractions with an enzymatic activity. The resultant purified non-reducing saccharide-forming enzyme was a protein with a specific activity of about 195 units/mg protein, and the yield was about 220 units per one L of the culture.

Throughout the present specification, the non-reducing saccharide-forming enzyme activity is assayed by the following method and expressed by the value of units: Place in a test tube 4 ml of 50 mM phosphate buffer (pH 7.0) containing 1.25 w/v % maltopentaose, add one ml of an enzyme solution to the tube, incubate the mixture at 40°C for 60 min to effect enzymatic reaction, and heat the resultant mixture at 100°C for 10 min to suspend the reaction. Dilute the reaction mixture with distilled water by 10 fold, and assay the reducing power by the Somogyi-Nelson's method. As a control, an enzyme solution, preheated at 100°C for 10 min to inactivate the enzyme, was treated similarly as above. One unit activity of the non-reducing saccharide-forming enzyme is defined as the amount of enzyme which reduces the reducing power corresponding to one  $\mu$ mol of maltopentaose per min.

##### Experiment 1-2

##### Preparation of trehalose-releasing enzyme

One hundred ml aliquots of a liquid nutrient culture medium (pH 7.0) containing 2.0 w/v % "PINE-DEX #4", a partial starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Hyogo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate, and 0.1 w/v % sodium dihydrogen phosphate were placed in 500-ml Erlenmeyer flasks, and sterilized by autoclaving at 120°C for 20 min. After cooling the flasks, a seed of *Rhizobium* sp. M-11 (FERM BP-4130) was inoculated into each medium and incubated at 27°C for 24 hours under rotatory shaking conditions to obtain a seed culture. Thereafter, 20 L aliquots of a fresh preparation of the same medium

were placed in 30-L jar fermenters, sterilized, and inoculated with one v/v of the seed culture, followed by the incubation at a pH of 6-8 and at 30°C for 24 hours under aeration-agitation conditions.

An about 18 L of the resultant culture was treated similarly as in Experiment 1-1 to disrupt cells, and the suspension was purified to obtain a trehalose-releasing enzyme with a specific activity of about 240 units/mg protein. The yield was about 650 units per one L of the culture.

Throughout the present specification, the trehalose-releasing enzyme activity is assayed by the following method and expressed by the value of units: Place in a test tube 4 ml of 50 mM phosphate buffer (pH 7.0) containing 1.25 w/v %  $\alpha$ -maltotriosyltrehalose, add one ml of an enzyme solution to the tube, and incubate the mixture at 40°C for 30 min to effect enzymatic reaction. One ml of the reaction mixture was placed in a test tube, add 2 ml of Somogyi copper solution to the mixture to suspend the reaction, and assay the reducing power by the Somogyi-Nelson's method. As a control, an enzyme solution, preheated at 100°C for 10 min to inactivate the enzyme, was treated similarly as above. One unit activity of the trehalose-releasing enzyme is defined as the amount of enzyme which increases the reducing power corresponding to one  $\mu$ mol of glucose per min.

## Experiment 2

### Preparation of trehalose

#### Experiment 2-1

##### Preparation of anhydrous crystalline trehalose

One part by weight of potato starch was suspended in 10 parts by weight of water and mixed with a bacterial liquefying  $\alpha$ -amylase in usual manner, followed by heating the mixture to 90°C to gelatinize and liquefy the contents up to give a DE (dextrose equivalent) 0.5, then immediately heating the resultant to 130°C to suspend the enzymatic reaction. The liquefied starch was promptly cooled to 45°C, then mixed with one unit/g starch, d.s.b., of the non-reducing saccharide-forming enzyme obtained in Experiment 1-1, one unit/g starch, d.s.b., of the trehalose releasing enzyme obtained in Experiment 1-2, and 200 units/g starch, d.s.b., of an isoamylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, followed by saccharifying the contents for 48 hours while keeping the pH at about 6.0 to obtain a reaction mixture containing 80.5 w/w % trehalose, d.s.b. The reaction mixture was in usual manner decolorized with an activated charcoal, desalted and purified with an ion exchanger, concentrated up to 75 w/w %, transferred to a crystallizer, heated to 50°C, mixed with one w/w %, d.s.b., of crystalline trehalose hydrate, and cooled to 30°C during 24 hours under gently stirring conditions. The resultant masseccuite containing the crystalline trehalose hydrate was separated with a basket-type centrifuge, and the collected crystal was sprayed with a small amount of water to obtain crystalline trehalose hydrate containing 99.0 w/w % trehalose in a yield of 47% to the material starch, d.s.b.

#### Experiment 2-2

##### Preparation of anhydrous crystalline trehalose

A portion of the crystalline trehalose hydrate obtained by the method in Experiment 2-1 was provided, dissolved in a small amount of water by heating, transferred to a vessel, and concentrated into a syrup with a moisture content of 9.5 w/w % under a reduced pressure. The syrup was placed in a crystallizer, mixed with as a seed crystal one w/w % of anhydrous crystalline trehalose, d.s.b., and crystallized at 100°C for 5 min under stirring conditions. The resultant masseccuite was distributed into plastic plain vessels and aged by standing at 70°C for 3 hours. Thereafter, the solidified masseccuites in a block form were removed from the vessels and pulverized in usual manner to obtain a powdery anhydrous crystalline trehalose with a moisture content of about one w/w % in a yield of about 90% to the material, d.s.b.

#### Experiment 2-3

##### Preparation of anhydrous amorphous trehalose

A portion of the crystalline trehalose hydrate obtained by the method in Experiment 2-1 was dissolved in water to give a concentration of about 40 w/w %, lyophilized, pulverized to obtain an anhydrous amorphous trehalose powder with a moisture content of about 2 w/w % in a yield of about 100% to the material, d.s.b.

Experiment 3Preparation of octaacetyltrehalose

5        Sixty-five g dry pyridine containing 50 g acetic anhydride was placed in a reaction container, cooled to 0°C, and mixed with 3 g either of the crystalline trehalose hydrate, anhydrous crystalline trehalose, and anhydrous amorphous trehalose obtained in Experiment 2-1 to 2-3, respectively, followed by completely dissolving the saccharide in the solvent under gentle stirring conditions. The solution was allowed to stand at ambient temperature for 18 hours to react the contents, and the reaction mixture was poured into iced water, allowed to stand for a while, and separated by  
10        decantation to collect a layer of the organic solvent, followed by concentrating the layer. The concentrate was analyzed by conventional method using gas chromatography to quantify the formed octaacetyltrehalose, followed by macroscopically observing the color of the octaacetyltrehalose. The results were in Table.

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Table

Substrate	Yield of octaacetyl trehalose (%)	Degree of color	Judgement
Crystalline trehalose hydrate	60	+ +	Control
Anhydrous crystalline trehalose	98	±	Present invention
Anhydrous amorphous trehalose	97	±	Present invention

Note : In Table, the symbol "±" means that a product slightly colored, and the symbol "++" means that a product strongly colored when observed macroscopically.

The results in Table show that a strongly colored octaacetyl trehalose is obtained with a theoretical yield of 60%

when used crystalline trehalose hydrate as a substrate, while a high quality octaacetylthrehalose is obtained with almost a theoretical yield when used anhydrous crystalline trehalose and anhydrous amorphous trehalose as substrates. The data show that the present invention using anhydrous trehalose as a substrate significantly improves the yield and quality of trehalose derivatives as compared with those obtained by the methods using crystalline trehalose hydrate.

5 The following examples explain the present invention:

#### Example 1

##### Trehalose linoleate

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Two hundred ml of anhydrous pyridine and the anhydrous amorphous crystalline trehalose, obtained by the method in Experiment 2-3, were placed in a reaction container, and mixed with 4 g of thiazolithione-linoleic acid amide dissolved in 5 ml anhydrous pyridine. To the reaction mixture was added 85 mg of 60 w/w % oily sodium hydride, and the resultant was reacted at ambient temperature for 2 hours. The reaction mixture was admixed with 1.5 ml of saturated aqueous ammonium chloride solution, followed by removing pyridine *in vacuo* to obtain 8.5 g residue. The purification of the residue using silica gel chromatography resulted in 5.3 g trehalose linoleate with an average substitution degree of 1.4.

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The tasteless and odorless product with a considerably high activity can be arbitrarily used in foods, cosmetics and pharmaceuticals as a safety non-ionic surfactant. As a control, the crystalline trehalose hydrate obtained by the method in Experiment 2-1 was reacted similarly as above, resulting in only 2.3 g strongly-colored trehalose linoleate.

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#### Example 2

##### Trehalose myristate

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Two hundred and twenty g of anhydrous crystalline trehalose, obtained by the method in Experiment 2-2, was dissolved in 800 ml of N,N'-dimethylformamide, admixed with 60 g of myristic acid methylester and 4 g of calcium carbonate, and subjected to an enzymatic reaction at a temperature of 85-95°C for 24 hours under a reduced pressure of 100-200 mmHg. Thereafter, the organic solvent was removed from the reaction mixture *in vacuo*, and the residue was soaked twice in 300 ml acetone. The extracts were pooled and washed with benzene and oil ether to obtain a viscous oily product which was then soaked again in 300 ml acetone. The extract thus obtained was cooled, and the formed sediment was collected and dried into 310 g trehalose myristate with an average substitution degree of 1.7.

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The tasteless and odorless product with a considerably high activity can be arbitrarily used in foods, cosmetics and pharmaceuticals as a safety non-ionic surfactant. Because the product has an activity of inhibiting the growth of malignant tumors, it can be also used as an effective ingredient for pharmaceuticals. As a control, the crystalline trehalose hydrate, obtained by the method in Experiment 2-1, was reacted similarly as above to obtain only 90 g strongly-colored trehalose myristate.

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#### Example 3

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##### Trehalose dodecyl ether

Three hundred and ninety g of n-dodecanol was placed in a reaction container, heated to 125°C, and admixed with one g of p-toluene sulfonate as a catalyst, followed by reducing the inner pressure of the container to 5-10 mmHg. One hundred g of anhydrous amorphous trehalose, obtained by the method in Experiment 2-3, was suspended in 130 g n-dodecanol, and the suspension was added to the container drop by drop at a rate of 2.3 g/min during 100 min. The resultant reaction mixture was neutralized with saturated sodium carbonate, followed by removing intact n-dodecanol to obtain about 140 g of a composition containing 79.9 w/w % trehalose dodecyl ether, d.s.b.

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The product with a relatively high activity can be arbitrarily used as a safety surfactant in detergents for laundry and kitchen, and those in general including shampoos. As a control, crystalline trehalose hydrate, obtained by the method in Experiment 2-1, was reacted similarly as above, resulting in an about 80 g composition containing 27.5 w/w % strongly-colored trehalose dodecyl ether, d.s.b.

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#### Example 4

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##### Trehalose sulfate

One part by weight of anhydrous amorphous trehalose, obtained by the method in Experiment 2-3, was placed in a reaction container, and mixed drop by drop with 5 parts by weight of sulfur trioxide/dimethylformamide complex under



the stream of nitrogen gas. The mixture was successively subjected to an enzymatic reaction at ambient temperature for 4 hours and at 70°C for one hour. The reaction mixture was neutralized with an adequate amount of 5 N sodium hydroxide, mixed with 5-fold volumes of methyl alcohol, and allowed to stand for a while, followed by collecting the formed sediment by filtration by means of suction to obtain trehalose sulfate with an average substitution degree of 7.7 in a yield of about 95 w/w %, d.s.b.

The product with a relatively-high quality can be used in cosmetics in general such as humectants and skin-beautifying agents. As a control, crystalline trehalose hydrate, obtained by the method in Experiment 2-1, was subjected to an enzymatic reaction similarly as above to obtain strongly-colored trehalose sulfate with an average substitution degree of 6.5 in a yield of about 63 w/w %, d.s.b.

#### Example 5

##### Trehalose sulfate

#### Example 5-1

##### Preparation of maltose/trehalose converting enzyme

To 500-ml flasks were added 100 ml aliquots of a liquid nutrient culture medium (pH 7.2) consisting of 2.0 w/v % glucose, 0.5 w/v % polypeptone, 0.1 w/v % yeast extract, 0.1 w/v % dipotassium hydrogen phosphate, 0.06 w/v % sodium dihydrogen phosphate, 0.05 w/v % magnesium sulfate heptahydrate, 0.5 w/v % calcium carbonate, and water, and the flasks were sterilized by heating at 115°C for 30 min, cooled, inoculated with a seed of *Pimerobacter* sp. R48 (FERM BP-4315), followed by the cultivation at 27°C for 24 hours under a stirring condition of 200 rpm to obtain a seed culture. Thereafter, 20-L aliquots of a fresh preparation of the same liquid nutrient culture medium were placed in 30-L jar fermenters, sterilized similarly as above, cooled to 27°C, inoculated with one v/v % of the seed culture, and cultured at a pH of 6.0-8.0 and at 27°C for 40 hours under aeration-agitation conditions.

The resultant culture was centrifuged, and about 0.5 kg of the collected wet cells were suspended in 10 mM phosphate buffer (pH 7.0) and disrupted in usual manner, followed by centrifuging the resultant suspension to obtain an about 4.5 L crude enzyme solution. Ammonium sulfate was added to the suspension to give a saturation degree of about 30 w/v %, salted out by standing at 4°C for 4 hours, and centrifuged to obtain a supernatant. Ammonium sulfate was added to the supernatant to give a saturation degree of 80 w/v %, allowed to stand at 4°C overnight, and centrifuged to collect sediment which was then dissolved in a small amount of 10 mM phosphate buffer (pH 7.0), and dialyzed against 10 mM phosphate buffer (pH 7.0) for 24 hours. The dialyzed inner solution was centrifuged, and the supernatant was fed to a column packed with DEAE TOYOPEARL®, a resin for ion-exchange column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, and eluted with a linear gradient of sodium chloride increasing from 0 M to 0.4 M in 10 mM phosphate buffer (pH 7.0). From the eluate, fractions with the objective enzyme activity were collected, pooled, dialyzed against 10 mM phosphate buffer (pH 7.0) containing one M ammonium sulfate for 10 hours, and centrifuged to obtain a supernatant. The supernatant was fed to a column packed with "BUTYL TOYOPEARL®", a resin for hydrophobic column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been equilibrated with 10 mM phosphate buffer (pH 7.0) containing one M ammonium sulfate, and eluted with a linear gradient of ammonium sulfate decreasing from 1 M to 0 M in 10 mM phosphate buffer (pH 7.0). Fractions with an enzyme activity were collected from the eluate, pooled, fed to a column packed with "MONO Q HR5/5", an ion exchanger for ion exchange column chromatography commercialized by Pharmacia LKB Biotechnology AB Uppsala, Sweden, which had been equilibrated with 10 mM phosphate buffer (pH 7.0), and eluted from the column with a linear gradient of sodium chloride increasing from 0 M to 0.5 M in 10 mM phosphate buffer (pH 7.0), followed by collecting fractions with the objective enzyme activity. The resultant purified maltose/trehalose converting enzyme had a specific activity of about 17 units/mg protein in a yield of about 46 units per one L of the culture.

Throughout the present specification, the activity of the maltose/trehalose converting enzyme was assayed by the following method and expressed by the value of activity (unit): Place in a test tube one ml of 10 mM phosphate buffer (pH 7.0) containing 20 w/v % maltose, add one ml of an appropriately diluted enzyme solution to the tube, enzymatically react the contents by incubating at 25°C for 60 min, heat the reaction mixture at 100°C for 10 min to suspend the reaction. Dilute the reaction mixture with 50 mM phosphate buffer (pH 7.5) by 11-fold, place 0.4 ml of the diluted solution in a test tube, add 0.1 ml of a solution containing one unit/ml trehalase to the tube, incubate the tube at 45°C for 120 min, and quantify the glucose content in the reaction mixture by the glucose oxidase method. In parallel, provide as a control a system using an enzyme solution preheated at 100°C for 10 min to inactivate the enzyme, and treat the system similarly as above. Based on the quantified glucose content, estimate the formed trehalose content. One unit activity of the maltose/trehalose converting enzyme is defined as the amount that forms one  $\mu$ mol trehalose per min under the above conditions.

Example 5-2Preparation of anhydrous crystalline trehalose

5 Corn starch was suspended in water to give a concentration of 15 w/w %, adjusted to pH 5.5, mixed with 2 units/g starch, d.s.b., of "SPITASE HS", a liquefying  $\alpha$ -amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and heated at 90°C under stirring conditions to gelatinize and liquefy the starch. The liquefied starch was autoclaved at 120°C for 20 min to inactivate the enzyme, promptly cooled to 55°C, adjusted to pH 5.0, mixed with an isoamylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and a  $\beta$ -amylase specimen commercialized by Nagase Biochemicals, Ltd., Tokyo, Japan, in respective amounts of 300 units and 20 units per g starch, d.s.b., and allowed to react for 24 hours to obtain a reaction mixture containing 92 w/w % maltose, d.s.b. The reaction mixture was heated at 100°C for 20 min to inactivate the remaining enzyme, heated to 20°C, adjusted to pH 7.0, mixed with 1.5 units/g starch, d.s.b., of the maltose/trehalose converting enzyme obtained in Example 5-1, and enzymatically reacted for 72 hours to obtain a reaction mixture containing 71 w/w % trehalose, d.s.b. The resultant reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, cooled, purified similarly as in Experiment 2-1, and concentrated up to give a moisture content of 9.5 w/w %. The concentrate was placed in a crystallizer, admixed with one w/w %, d.s.b., of a powdery anhydrous crystalline trehalose as a seed, and crystallized at 110°C for 10 min under stirring conditions. The resultant masseccuite was distributed to plastic vessels and aged by standing at 70°C for 3 hours. Thereafter, the solidified masseccuite in a block form was removed from each vessels, pulverized in usual manner, and subjected to fluidized-bed drying to obtain a powdery anhydrous crystalline trehalose with a moisture content of about 2 w/w % in a yield of about 95% to the material starch, d.s.b.

Example 5-3Preparation of trehalose sulfate

One hundred g of anhydrous crystalline trehalose, obtained by the method in Example 5-2, was placed in a container, and sulfated by the method in Example 4 to obtain about 240 g of a composition containing trehalose sulfate with an average substitution degree of about 8.

30 The product with a relatively high quality can be arbitrarily used as a humectant or a skin-beautifying agent in cosmetics in general.

As is described above, the present invention enables to produce relatively high quality trehalose derivatives, which could not have been readily obtained by conventional methods, by reacting anhydrous trehalose with reactive reagents under anhydrous conditions. Unlike crystalline trehalose hydrate, anhydrous trehalose has no water of crystallization, so that it greatly reduces or even omits the drying step required for pre-reaction and leads to a considerably high reduction of the production cost of trehalose derivatives. The trehalose derivatives, obtained by the present invention, can be used in a variety of fields in the production, the chemical synthesis, and the enzymatic synthesis of foods, cosmetics, pharmaceuticals, detergents and chemicals as surfactants, humectants, skin-beautifying agents, antitumor agents, and intermediates for chemical and enzymatic syntheses.

40 While there has been described what is at present considered to be the preferred embodiments of the invention, it will be understood the various modifications may be made therein, and it is intended to cover in the appended claims all such modifications as fall within the true spirit and scope of the invention.

Claims

1. A process for producing a trehalose derivative, which comprises a step of allowing a reactive reagent to react with anhydrous trehalose under anhydrous conditions.
- 50 2. A process according to claim 1, wherein said anhydrous trehalose is anhydrous crystalline- or anhydrous amorphous-trehalose.
3. A process according to claim 1 or claim 2, wherein said anhydrous trehalose contains at least 70 w/w % trehalose, on a dry solid basis.
- 55 4. A process according to any one of claims 1 to 3, wherein said anhydrous trehalose contains a moisture less than 3 w/w % when determined by the Karl Fischer's method.

5. A process according to any one of the preceding claims, wherein said anhydrous trehalose is obtained by allowing a non-reducing saccharide-forming enzyme to act on a partial starch hydrolysate to form a non-reducing saccharide having a trehalose structure as an end unit, allowing a trehalose-releasing enzyme to act on said non-reducing saccharide to release trehalose, and drying or crystallizing the released trehalose.

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6. A process according to any one of claims 1 to 4, wherein said anhydrous trehalose is obtained by allowing a maltose/trehalose converting enzyme to act on maltose to form trehalose, and drying or crystallizing the formed trehalose.

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7. A process according to any one of the preceding claims, wherein said reactive reagent is a member selected from the group consisting of acids, salts, alcohols, ketones, halogens, amines, and reactive derivatives thereof.

8. A process according to any one of the preceding claims, wherein said trehalose derivative is in the form of an ester or an ether.

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/EP92/02809  <b>(22) International Filing Date:</b> 4 December 1992 (04.12.92)  <b>(30) Priority data:</b> 9126405.1 12 December 1991 (12.12.91) GB 9202522.0 6 February 1992 (06.02.92) GB  <b>(71) Applicant (for all designated States except US):</b> GLAXO GROUP LIMITED [GB/GB]; Glaxo House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> AKEHURST, Rachel Ann [GB/GB]; TAYLOR, Anthony, James [GB/GB]; WYATT, David, Andrew [GB/GB]; Glaxo Group Research Limited, Park Road, Ware, Hertfordshire SG12 0DP (GB).		<b>(74) Agents:</b> FILLER, Wendy, Anne et al.; Glaxo Holdings plc, Glaxo House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <i>Equivalent of EP 0616524</i>
<b>(54) Title:</b> MEDICAMENTS  <b>(57) Abstract</b>  This invention relates to aerosol formulations of use for the administration of medicaments by inhalation, in particular a pharmaceutical aerosol formulation which comprises particulate medicament and a fluorocarbon or hydrogen-containing chlorofluorocarbon propellant, which formulation is substantially free of surfactant and with the proviso that said medicament is other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof. A method of treating respiratory disorders which comprises administration by inhalation of an effective amount of a pharmaceutical aerosol formulation as defined is also described.		

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## MEDICAMENTS

This invention relates to aerosol formulations of use for the administration of  
5 medicaments by inhalation.

The use of aerosols to administer medicaments has been known for several decades. Such aerosols generally comprise the medicament, one or more chlorofluorocarbon propellants and either a surfactant or a solvent, such as ethanol. The most commonly used aerosol propellants for medicaments have been propellant 11 ( $\text{CCl}_3\text{F}$ ) and/or  
10 propellant 114 ( $\text{CF}_2\text{ClCF}_2\text{Cl}$ ) with propellant 12 ( $\text{CCl}_2\text{F}_2$ ). However these propellants are now believed to provoke the degradation of stratospheric ozone and there is thus a need to provide aerosol formulations for medicaments which employ so called "ozone-friendly" propellants.

A class of propellants which are believed to have minimal ozone-depleting effects in  
15 comparison to conventional chlorofluorocarbons comprise fluorocarbons and hydrogen-containing chlorofluorocarbons, and a number of medicinal aerosol formulations using such propellant systems are disclosed in, for example, EP 0372777, WO91/04011, WO91/11173, WO91/11495 and WO91/14422. These applications are all concerned with the preparation of pressurised aerosols for the administration of  
20 medicaments and seek to overcome the problems associated with the use of the new class of propellants, in particular the problems of stability associated with the pharmaceutical formulations prepared. The applications all propose the addition of one or more of adjuvants such as alcohols, alkanes, dimethyl ether, surfactants (including fluorinated and non-fluorinated surfactants, carboxylic acids, polyethoxylates etc) and even conventional  
25 chlorofluorocarbon propellants in small amounts intended to minimise potential ozone damage.

Thus, for example EP 0372777 requires the use of 1,1,1,2-tetrafluoroethane in combination with both a cosolvent having greater polarity than 1,1,1,2-tetrafluoroethane (e.g. an alcohol or a lower alkane) and a surfactant in order to achieve a stable  
30 formulation of a medicament powder. In particular it is noted in the specification at page

which is substantially completely insoluble in the selected propellant. Appropriate medicaments may thus be selected from, for example, analgesics, e.g. codeine, dihydromorphine, ergotamine, fentanyl or morphine; anginal preparations, e.g. diltiazem; antiallergics, e.g. cromoglycate, ketotifen or nedocromil; anti-infectives, e.g. cephalosporins, penicillins, streptomycin, sulphonamides, tetracyclines and pentamidine; antihistamines, e.g. methapyrilene; anti-inflammatories, e.g. flunisolide, budesonide, tiptredane or triamcinolone acetonide; antitussives, e.g. noscapine; bronchodilators, e.g. ephedrine, adrenaline, fenoterol, formoterol, isoprenaline, metaproterenol, phenylephrine, phenylpropanolamine, pirbuterol, reproterol, rimiterol, terbutaline, isoetharine, tulobuterol, orciprenaline, or (-)-4-amino-3,5-dichloro- $\alpha$ -[[[6-[2-(2-pyridinyl) ethoxy] hexyl]amino]methyl]benzenemethanol; diuretics, e.g. amiloride; anticholinergics e.g. ipratropium, atropine or oxitropium; hormones, e.g. cortisone, hydrocortisone or prednisolone; xanthines e.g. aminophylline, choline theophyllinate, lysine theophyllinate or theophylline; and therapeutic proteins and peptides, e.g. insulin or glucagon. It will be clear to a person skilled in the art that, where appropriate, the medicaments may be used in the form of salts (e.g. as alkali metal or amine salts or as acid addition salts) or as esters (e.g. lower alkyl esters) or as solvates (e.g. hydrates) to optimise the activity and/or stability of the medicament and/or to minimise the solubility of the medicament in the propellant.

Particularly preferred medicaments for administration using aerosol formulations in accordance with the invention include anti-allergics, bronchodilators and anti-inflammatory steroids of use in the treatment of respiratory disorders such as asthma by inhalation therapy, for example cromoglycate (e.g. the sodium salt), terbutaline (e.g. the sulphate salt), reproterol (e.g. the hydrochloride salt) or (-)-4-amino-3,5-dichloro- $\alpha$ -[[[6-[2-(2-pyridinyl)-ethoxy]hexyl]amino]methyl]benzenemethanol.

It will be appreciated by those skilled in the art that the aerosol formulations according to the invention may, if desired, contain a combination of two or more active ingredients. Aerosol compositions containing two active ingredients (in a conventional propellant system) are known, for example, for the treatment of respiratory disorders such

as asthma. Accordingly the present invention further provides aerosol formulations in accordance with the invention which contain two or more particulate medicaments. Medicaments may be selected from suitable combinations of the medicaments mentioned hereinbefore. Thus, suitable combinations of bronchodilatory agents include ephedrine  
5 and theophylline, fenoterol and ipratropium, and isoetharine and phenylephrine aerosol formulations.

Preferred aerosol formulations in accordance with the invention comprise (a) an effective amount of a particulate bronchodilatory medicament (b) an effective amount of a particulate antiinflammatory, preferably a steroidal antiinflammatory medicament and (c) a  
10 fluorocarbon or hydrogen - containing chlorofluorocarbon propellant with the proviso that said medicaments are other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof. Alternatively aerosol formulations may contain a bronchodilator such as isoprenaline in combination with an antiallergic such as cromoglycate (e.g. the sodium salt).  
15 Combinations of isoprenaline and sodium cromoglycate are especially preferred.

The final aerosol formulation desirably contains 0.005-10% w/w, preferably 0.005-5% w/w, especially 0.01-1.0% w/w, of medicament relative to the total weight of the formulation.

The propellants for use in the invention may be any fluorocarbon or  
20 hydrogen-containing chlorofluorocarbon or mixtures thereof having a sufficient vapour pressure to render them effective as propellants. Preferably the propellant will be a non-solvent for the medicament. Suitable propellants include, for example, C<sub>1-4</sub>hydrogen-containing chlorofluorocarbons such as CH<sub>2</sub>ClF, CClF<sub>2</sub>CHClF, CF<sub>3</sub>CHClF, CHF<sub>2</sub>CClF<sub>2</sub>, CHClFCHF<sub>2</sub>, CF<sub>3</sub>CH<sub>2</sub>Cl and CClF<sub>2</sub>CH<sub>3</sub>; C<sub>1-4</sub>hydrogen-containing  
25 fluorocarbons such as CHF<sub>2</sub>CHF<sub>2</sub>, CF<sub>3</sub>CH<sub>2</sub>F, CHF<sub>2</sub>CH<sub>3</sub> and CF<sub>3</sub>CHF<sub>2</sub>CF<sub>3</sub>; and perfluorocarbons such as CF<sub>3</sub>CF<sub>3</sub> and CF<sub>3</sub>CF<sub>2</sub>CF<sub>3</sub>.

Where mixtures of the fluorocarbons or hydrogen-containing chlorofluorocarbons are employed they may be mixtures of the above identified compounds or mixtures, preferably binary mixtures, with other fluorocarbons or hydrogen-containing  
30 chlorofluorocarbons for example CHClF<sub>2</sub>, CH<sub>2</sub>F<sub>2</sub> and CF<sub>3</sub>CH<sub>3</sub>. Preferably a single



fluorocarbon or hydrogen-containing chlorofluorocarbon is employed as the propellant. Particularly preferred as propellants are C<sub>1-4</sub> hydrogen-containing fluorocarbons such as 1,1,1,2-tetrafluoroethane (CF<sub>3</sub>CH<sub>2</sub>F) and 1,1,1,2,3,3,3-heptafluoro-n-propane (CF<sub>3</sub>CHF<sub>2</sub>CF<sub>3</sub>).

5 It is desirable that the formulations of the invention contain no components which may provoke the degradation of stratospheric ozone. In particular it is desirable that the formulations are substantially free of chlorofluorocarbons such as CCl<sub>3</sub>F, CCl<sub>2</sub>F<sub>2</sub> and CF<sub>3</sub>CCl<sub>3</sub>.

10 The propellant may additionally contain a volatile adjuvant such as a saturated hydrocarbon for example propane, n-butane, isobutane, pentane and isopentane or a dialkyl ether for example dimethyl ether. In general, up to 50% w/w of the propellant may comprise a volatile hydrocarbon, for example 1 to 30% w/w. However, formulations which are substantially free of volatile adjuvants are preferred.

15 It is further desirable that the formulations of the invention are substantially free of liquid components of higher polarity than the propellant employed. Polarity may be determined for example, by the method described in European Patent Application Publication No. 0327777. In particular formulations which are substantially free of alcohols such as ethanol are preferable. As used herein "substantially free" means less than 1% w/w based upon the fluorocarbon or hydrogen-containing chlorofluorocarbon, in particular less than 0.5% for example 0.1% or less.

20 A particularly preferred embodiment the invention provides a pharmaceutical aerosol formulation consisting essentially of one more particulate medicament and one or more fluorocarbon or hydrogen-containing chlorofluorocarbon propellant, with the proviso that said medicament is other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof.

25 The formulations of the invention may be prepared by dispersal of the medicament in the selected propellant in an appropriate container, e.g. with the aid of sonication. The process is desirably carried out under anhydrous conditions to obviate any adverse effects of moisture on suspension stability.

Optionally, the medicament may be surface-modified prior to its dispersion in the propellant by treatment with a substantially non-polar liquid medium which is a non-solvent for the medicament. There is thus provided in a further aspect of the invention an aerosol formulation comprising particulate, surface-modified medicament, as defined herein, and a fluorocarbon or hydrogen-containing chlorofluorocarbon propellant, which formulation is substantially free of surfactant. By "surface-modified medicament" is meant particles of medicament which have been surface-modified by admixture with a substantially non-polar non-solvent liquid, followed by removal of the liquid, with the proviso that said medicament is other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof. The substantially non-polar non-solvent liquid medium is conveniently an aliphatic hydrocarbon, e.g. a lower alkane, which is sufficiently volatile to permit its ready evaporation, e.g. at ambient temperature and pressure, after slurring with the medicament. The use of isopentane as liquid medium is particularly advantageous in this respect.

The medicament is desirably slurried with the liquid medium under anhydrous conditions to obviate any adverse effects of moisture on suspension stability. The slurry may advantageously be sonicated to maximise the surface-modifying effect of the treatment. The liquid may be removed by any convenient means for example by evaporation or by filtration followed by evaporation, provided that following treatment the medicament is substantially free of the liquid. The formulations of the invention will be substantially free of the non-solvent non-polar liquid. Surface-modified medicament prepared by the above described process comprises a further aspect of the present invention.

The formulations according to the invention may be filled into canisters suitable for delivering pharmaceutical aerosol formulations. Canisters generally comprise a container capable of withstanding the vapour pressure of the propellant used such as a plastic or plastic-coated glass bottle or preferably a metal can, for example an aluminium can which may optionally be anodised, lacquer-coated and/or plastic-coated, which container is closed with a metering valve. The metering valves are designed to deliver a metered

amount of the formulation per actuation and incorporate a gasket to prevent leakage of propellant through the valve. The gasket may comprise any suitable elastomeric material such as for example low density polyethylene, chlorobutyl, black and white butadiene-acrylonitrile rubbers, butyl rubber and neoprene. Suitable valves are commercially available from manufacturers well known in the aerosol industry, for example, from Valois, France (e.g. DF10, DF30, DF60), Bepak plc, UK (e.g. BK300, BK356) and 3M-Neotechnic Ltd, UK (e.g. Spraymiser™).

Conventional bulk manufacturing methods and machinery well known to those skilled in the art of pharmaceutical aerosol manufacture may be employed for the preparation of large scale batches for the commercial production of filled canisters. Thus, for example, in one bulk manufacturing method a metering valve is crimped onto an aluminium can to form an empty canister. The particulate medicament is added to a charge vessel and liquified propellant is pressure filled through the charge vessel into a manufacturing vessel. The drug suspension is mixed before recirculation to a filling machine and an aliquot of the drug suspension is then filled through the metering valve into the canister. Typically, in batches prepared for pharmaceutical use, each filled canister is check-weighed, coded with a batch number and packed into a tray for storage before release testing.

Each filled canister is conveniently fitted into a suitable channelling device prior to use to form a metered dose inhaler for administration of the medicament into the lungs or nasal cavity of a patient. Suitable channelling devices comprise for example a valve actuator and a cylindrical or cone-like passage through which medicament may be delivered from the filled canister via the metering valve to the nose or mouth of a patient e.g. a mouthpiece actuator. Metered dose inhalers are designed to deliver a fixed unit dosage of medicament per actuation or "puff", for example in the range of 10 to 5000 microgram medicament per puff.

Administration of medicament may be indicated for the treatment of mild, moderate or severe acute or chronic symptoms or for prophylactic treatment. It will be appreciated that the precise dose administered will depend on the age and condition of the patient, the particular particulate medicament used and the frequency of administration and will

ultimately be at the discretion of the attendant physician. When combinations of medicaments are employed the dose of each component of the combination will in general be that employed for each component when used alone. Typically, administration may be one or more times, for example from 1 to 8 times per day, giving for example 1,2,3 or 4 puffs each time.

Thus, for example, each valve actuation may deliver 5mg sodium cromoglycate, 250 microgram terbutaline sulphate or 500 microgram reproterol hydrochloride. Typically each filled canister for use in a metered dose inhaler contains 100, 160 or 240 metered doses or puffs of medicament.

The filled canisters and metered dose inhalers described herein comprise further aspects of the present invention.

A still further aspect of the present invention comprises a method of treating respiratory disorders such as, for example, asthma, which comprises administration by inhalation of an effective amount of a formulation as herein described.

The following non-limitative Examples serve to illustrate the invention.

#### Example 1

Micronised sodium cromoglycate (1.2g) is weighed directly into an aluminium can and 1,1,1,2-tetrafluorethane (to 18.2g) added from a vacuum flask. A metering valve is crimped into place and the sealed can sonicated for five minutes. The aerosol delivers 5mg sodium cromoglycate per actuation.

#### Example 2

Micronised terbutaline sulphate (60mg) is weighed directly into an aluminium can and 1,1,1,2-tetrafluorethane (to 18.2g) added from a vacuum flask. A metering valve is crimped into place and the sealed can sonicated for five minutes. The aerosol delivers 250 microgram terbutaline sulphate per actuation.

**Claims**

1. A pharmaceutical aerosol formulation which comprises particulate medicament and a fluorocarbon or hydrogen-containing chlorofluorocarbon propellant, which formulation  
5 is substantially free of surfactant and with the proviso that said medicament is other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof.
2. A pharmaceutical aerosol formulation consisting essentially of one more particulate  
10 medicament and one or more fluorocarbon or hydrogen-containing chlorofluorocarbon propellant, with the proviso that said medicament is other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof.
- 15 3. A formulation as claimed in Claim 1 or Claim 2 wherein said medicament is an anti-allergic, a bronchodilator or an anti-inflammatory steroid.
4. A formulation as claimed in any one of Claims 1 to 3 wherein said medicament is cromoglycate, terbutaline, reproterol or (-)-4-amino-3,5-dichloro- $\alpha$ -[[[6-[2-  
20 (2-pyridinyl)ethoxy]hexyl]amino]methyl]benzenemethanol or a physiologically acceptable salt thereof.
5. A formulation as claimed in any one of claims 1 to 4 which contains two or more  
25 particulate medicaments.
6. A formulation as claimed in any one of claims 1 to 5 which comprises a particulate bronchodilatory medicament and a particulate anti-inflammatory medicament.

7. A formulation as claimed in any one of Claims 1 to 5 which comprises a particulate bronchodilatory medicament and a particulate anti-allergic medicament.
8. A formulation as claimed in Claim 7 which comprises isoprenaline and cromoglycate or a physiologically acceptable salt thereof.
9. A formulation as claimed in any one of Claims 1 to 8 which contains 0.005 to 10% w/w of medicament relative to the total weight of the formulation.
10. A formulation as claimed in any one of Claims 1 to 9 wherein the propellant is 1,1,1,2-tetrafluoroethane or 1,1,1,2,3,3,3-heptafluoro-n-propane.
11. A formulation as claimed in any one of Claims 1 to 10 which has a respirable fraction of 20% or more by weight of the medicament.
12. A formulation as claimed in any one of Claims 1 to 11 wherein said particulate medicament is surface-modified.
13. Surface-modified medicament prepared by admixture of particles of a medicament other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof, with a substantially non-polar, non-solvent liquid, followed by removal of the liquid.
14. A canister suitable for delivering a pharmaceutical aerosol formulation which comprises a container capable of withstanding the vapour pressure of the propellant used, which container is closed with a metering valve and contains a pharmaceutical aerosol formulation which comprises particulate medicament and a fluorocarbon or hydrogen-containing chlorofluorocarbon propellant, which formulation is substantially free of surfactant and with the proviso that said medicament is other than salmeterol,

salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof.

15. A metered dose inhaler which comprises a canister as claimed in Claim 14 fitted into a suitable channelling device.

16. A method of treating respiratory disorders which comprises administration by inhalation of an effective amount of a pharmaceutical aerosol formulation which comprises particulate medicament and a fluorocarbon or hydrogen-containing chlorofluorocarbon propellant, which formulation is substantially free of surfactant and with the proviso that said medicament is other than salmeterol, salbutamol, fluticasone propionate, beclomethasone dipropionate or a physiologically acceptable salt or solvate thereof.

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 92/02809

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K9/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	A61K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	WO,A,9 104 011 (RIKER) 4 April 1991 cited in the application see claims see page 6, line 27 - line 32 see page 8, line 1 - line 22 ---	1-16
A	WO,A,9 111 495 (BOEHRINGER INGELHEIM) 8 August 1991 cited in the application see claims see page 2, line 10 - line 31 ---	1-16
A	WO,A,9 111 173 (FISONS) 8 August 1991 cited in the application see claims see page 3, line 15 - line 16 ---	1-16
-/--		
<p><sup>10</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
09 MARCH 1993		23. 03. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		SCARPONI U.



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 92/02809

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**ALTHOUGH CLAIM 16 IS DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN BODY BY THERAPY (RULE 39.1(IV) PCT) THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOSITION.**
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9202809  
SA 67186

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 09/03/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9104011	04-04-91	AU-A- 6409790	18-04-91
		EP-A- 0493437	08-07-92
WO-A-9111495	08-08-91	DE-A- 4003272	08-08-91
		AU-A- 7211391	21-08-91
		EP-A- 0514415	25-11-92
WO-A-9111173	08-08-91	EP-A- 0513127	19-11-92
WO-A-9114422	03-10-91	AU-A- 7668691	21-10-91
		EP-A- 0526481	10-02-93
		US-A- 5118494	02-06-92
WO-A-9208447	29-05-92	AU-A- 8862991	11-06-92